GP1632

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In re Application of: Aebersold et al.

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For: RAPID QUANTITATIVE ANALYSIS OF PROTEINS OR PROTEIN FUNCTION IN COMPLEX MIXTURES

CERTIFICATE OF MAILING

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REQUEST FOR RE-PUBLICATION OF PATENT APPLICATION UNDER 37 C.F.R. § 1.221(b)

Asst. Commissioner for Patents
BOX PG PUB
Washington, D.C. 20231

Sir:

Applicants request republication of the above-referenced application. Applicants believe that the publication errors listed below were made by the Patent Office.

In particular, the error in claim 11, citing "nitrites" instead of "nitriles" is a material mistake in that the two terms are very different chemical entities.

For your convenience, a copy of the marked-up paragraphs are provided.

In the claims:

Claim 11

Please replace "nitrites" with --nitriles--.

Page 5

Paragraph [0050]

Please replace "$COOH_l$" with --COOH,--.

Pages 11 - 12

Paragraph [0112]

Please replace "ineubated" with --incubated--.

Please replace two occurrences of "ineubation" with --incubation--.

Page 12

Paragraph [0113]

Please replace three occurrences of "ineubation" with --incubation--.

Paragraph [0114]

Please replace "ineubated" with --incubated--.

Please replace "p-galactosidase" with --β-galactosidase--.

Page 13

Paragraph [0126]

Please replace "0-acetyl" with --O-acetyl--.

Paragraph [0127]

Please replace "ineubation" with --incubation--.

Please replace "UDβ-GlcNAc" with --UDP-GlcNAC--.

Page 14

Paragraph [0130]

Please replace "ineubating" with --incubating--.

Paragraph [0131]

Please replace "Na'" with --Na^+--.

Paragraph [0139]

Please replace "ineubate" with --incubate--.

Paragraph [0141]

Please replace "ineubation" with --incubation--.

Page 15

Paragraph [0146]

Please replace "ineubated" with --incubated--.

Paragraph [0148]

Please insert a paragraph break before "Incubations were performed in a total of 30 mcL of total volume."

Paragraph [0149]

Please replace "ineubation" with --incubation--.

Paragraph [0151]

Please replace "ineubation/mg" with --incubation/mg--.

Paragraph [0154]

Please replace "(AG Mβ-50, Bio,Rad)" with --(AG MP-50, Bio,Rad)--.

Paragraph [0155]

Please replace "(0.1 5 mmol)" with --(0.15 mmol)--.

Page 16

Paragraph [0163]

Please replace "1,11 -Dicyano-3,6,9-trioxaundecane" with --1,11-Dicyano-3,6,9-trioxaundecane--.

Page 18

Paragraph [0180]

Please insert a paragraph break before "21. Deuterated analog of 23 (27) 20 mg .....".

Paragraph [0186]

Please replace "After2 hours" with --After 2 hours--.

Page 19

Paragraph [0190]

Please remove the paragraph break separating paragraphs [0190] and [0191].

Paragraph [0194]

Please delete the space between 1 and 2 in the first line.

Paragraph [0195]

Please delete the space between 1 and 3 in the first line.

Page 20

In Table 2

For the 2nd peptide of the LCA_BOVIN Gene, please replace "FLDDLTDDTMC#VK (SEQ ID NO:9)" with -- FLDDLTDDIMC#VK (SEQ ID NO:9)--.

Page 28

In Scheme 5

Remove the "F" in the para position.

In compound 4, please replace "n=1" second occurrence, with --n=0--.

In the last chemical structure, reverse O and ● in the occurrence nearest R^1.

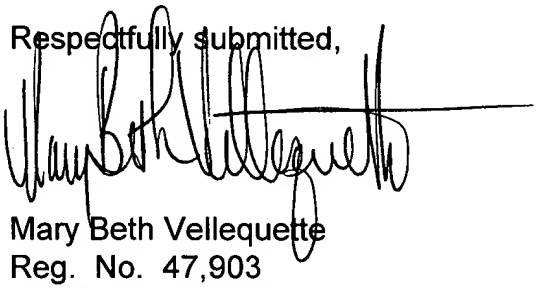
Page 30

In Scheme 10

Replace two occurrences of "Nac" with --NAc--.

It is believed this submission does not require the payment of a fee. If this is incorrect, please deduct any required fee from deposit account 07-1969.

Respectfully submitted,



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[0041] any 1,2-diol, such as 1,2-dihydroxyethane (HO—CH_2—CH_2—OH), and other 1,2-dihydroxyalkanes including those of cyclic alkanes, e.g., 1,2-dihydroxycyclohexane which bind to an alkyl or aryl boronic acid or boronic acid esters , such as phenyl-$B(OH)_2$ or hexyl-$B(OEthyl)_2$ which may be attached via the alkyl or aryl group to a solid support material, such as Agarose;

[0042] maltose which binds to maltose binding protein (as well as any other sugar/sugar binding protein pair or more generally to any ligand/ligand binding protein pairs that has properties discussed above);

[0043] a hapten, such as dinitrophenyl group, for any antibody where the hapten binds to an anti-hapten antibody that recognizes the hapten, for example the dinitrophenyl group will bind to an anti-dinitrophenyl-lgG;

[0044] a ligand which binds to a transition metal, for example, an oligomeric histidine will bind to Ni(II), the transition metal CR may be used in the form of a resin bound chelated transition metal, such as nitrilotriacetic acid-chelated Ni(II) or iminodiacetic acid-chelated Ni(II);

[0045] glutathione which binds to glutathione-S-transferase.

[0046] In general, any A-CR pair commonly used for affinity enrichment which meets the suitability criteria discussed above. Biotin and biotin-based affinity tags are preferred. Of particular interest are structurally modified biotins, such as d-iminobiotin, which will elute from avidin or strepavidin columns under solvent conditions compatible with ESI-MS analysis, such as dilute acids containing 10-20% organic solvent. It is expected that d-iminobiotin tagged compounds will elute in solvents below pH 4. d-Iminobiotin tagged protein reactive reagents can be synthesized by methods described herein for the corresponding biotin tagged reagents.

[0047] A displacement ligand, DL, is optionally used to displace A from CR. Suitable DLs are not typically present in samples unless added. DL should be chemically and enzymatically stable in the sample to be analyzed and should not react with or bind to components (other than CR) in samples or bind non-specifically to reaction vessel walls. DL preferably does not undergo peptide-like fragmentation during MS analysis, and its presence in sample should not significantly suppress the ionization of tagged peptide, substrate or reaction product conjugates.

[0048] DL itself preferably is minimally ionized during mass spectrometric analysis and the formation of ions composed of DL clusters is preferably minimal. The selection of DL, depends upon the A and CR groups that are employed. In general, DL is selected to displace A from CR in a reasonable time scale, at most within a week of its addition, but more preferably within a few minutes or up to an hour. The affinity of DL for CR should be comparable or stronger than the affinity of the tagged compounds containing A for CR. Furthermore, DL should be soluble in the solvent used during the elution of tagged compounds containing A from CR. DL preferably is free A or a derivative or structural modification of A. Examples of DL include, d-biotin or d-biotin derivatives, particularly those containing groups that suppress cluster formation or suppress ionization in MS.

[0049] The linker group (L) should be soluble in the sample liquid to be analyzed and it should be stable with respect to chemical reaction, e.g., substantially chemically inert, with components of the sample as well as A and CR groups. The linker when bound to A should not interfere with the specific interaction of A with CR or interfere with the displacement of A from CR by a displacing ligand or by a change in temperature or solvent. The linker should bind minimally or preferably not at all to other components in the system, to reaction vessel surfaces or CR. Any non-specific interactions of the linker should be broken after multiple washes which leave the A-CR complex intact. Linkers preferably do not undergo peptide-like fragmentation during $(MS)^n$ analysis. At least some of the atoms in the linker groups should be readily replaceable with stable heavy-atom isotopes. The linker preferably contains groups or moieties that facilitate ionization of the affinity tagged reagents, peptides, substrates or reaction products.

[0050] To promote ionization, the linker may contain acidic or basic groups, e.g., COOH or SO_3H, primary, secondary or tertiary amino groups, nitrogen-heterocycles, ethers, or combinations of these groups. The linker may also contain groups having a permanent charge, e.g., phosphonium groups, quaternary ammonium groups, sulfonium groups, chelated metal ions, tetralky or tetraryl borate or stable carbanions.

[0051] The covalent bond of the linker to A or PRG should typically not be unintentionally cleaved by chemical or enzymatic reactions during the assay. In some cases it may be desirable to cleave the linker from the affinity tag A or from the PRG, for example to facilitate release from an affinity column. Thus, the linker can be cleavable, for example, by chemical, thermal or photochemical reaction. Photocleavable groups in the linker may include the 1-(2-nitrophenyl)-ethyl group. Thermally labile linkers may, for example, be a double-stranded duplex formed from two complementary strands of nucleic acid, a strand of a nucleic acid with a complementary strand of a peptide nucleic acid, or two complementary peptide nucleic acid strands which will dissociate upon heating. Cleavable linkers also include those having disulfide bonds, acid or base labile groups, including among others, diarylmethyl or trimethylarylmethyl groups, silyl ethers, carbamates, oxyesters, thiesters, thionoesters, and α-fluorinated amides and esters. Enzymatically cleavable linkers can contain, for example, protease-sensitive amides or esters, β-lactamase-sensitive β-lactam analogs and linkers that are nuclease-cleavable, or glycosidase-cleavable.

[0052] The protein reactive group (PRG) can be a group that selectively reacts with certain protein functional groups or is a substrate of an enzyme of interest. Any selectively reactive protein reactive group should react with a functional group of interest that is present in at least a portion of the proteins in a sample. Reaction of PRG with functional groups on the protein should occur under conditions that do not lead to substantial degradation of the compounds in the sample to be analyzed. Examples of selectively reactive PRGs suitable for use in the affinity tagged reagents of this invention, include those which react with sulfhydryl groups to tag proteins containing cysteine, those that react with

the detection of low abundance membrane proteins and the relative quantity of tagged peptides tagged is maintained through the selective enrichment steps.

[0102] In the application of the methods of this invention to cell surface proteins, once the tagged proteins are fragmented, the tagged peptides behave no differently from the peptides generated from more soluble samples.

[0103] Synthesis of Affinity Tagged Protein Reactive Reagents That are Selective for Certain Proteins Groups

[0104] Synthetic routes exemplary affinity tagged reagents suitable for use in the methods of this invention are provided in Schemes 2-3 where well-known synthetic techniques are employed in synthesis of the non-deuterated and deuterated reagents.

[0105] Biotinyl-iodoacetylamidyl-4,7,10 trioxatridecanediamine 4 (Scheme 3) consists of a biotin group, a chemically inert spacer of capable of being isotopically labeled with stable isotopes and a iodoacetamidyl group, respectively. The biotin group is used for affinity enrichment of peptides derivatized with the reagent, the ethylene glycol linker is differentially isotopically labeled for mass spectral analysis and the iodoacetamidyl group provides specificity of the reagent for sulfhydryl-containing peptides. The reagent an be synthesized in an all hydrogen form (isotopically light form) with and with 1-20, and preferably 4-8 deuterium atoms in the linker (isotopically heavy forms).

[0106] Analysis of Velocities of Multiple Enzymes in Cell Lysates

[0107] Monitoring enzyme functions by biochemical assays is an essential diagnostic tool that employs a multitude of analytical techniques including spectrophotometric, fluorometric, and radiometric detection of products. However, current methods are difficult to use for assaying several enzymes simultaneously in a single sample. Mass spectrometry for quantification of a collection of metabolites in biological fluids has emerged as a powerful approach for the analysis of birth defects (Morris et al., 1994), but this analytical technique has not been developed for the direct analysis of rates of individual enzymatic steps. The analytical method described herein for monitoring and quantification of enzymatic activities in cell homogenates and other biological samples permits simultaneous (multiplex) monitoring of multiple reactions, and can be readily automated.

[0108] A feature of the method of this invention as applied to enzyme assays is the use of electrospray ionization mass spectrometry (ESI-MS) (Cole et al., 1997) for the simultaneous detection of enzymatic products and chemically identical internal standards, which are distinguished by stable isotope (deuterium) labeling. A second feature is the use of affinity tagged reagents containing an enzyme substrate which when combined with affinity purification provide for facile capture of enzymatic products from crude biological fluids. The affinity tagged reagents are designed to contain a target substrate for an enzyme of interest that is covalently attached to an affinity tag via a linker. Action of the enzyme of interest on the substrate conjugate causes cleavage or other modification that changes its molecular mass (Scheme 4). The change of mass is detected by ESI-MS. The linker and affinity tag used preferably facilitate ionization by ESI, block action of other enzymes in the biological fluid, and allow highly selective capture from the complex matrix for facile purification.

[0109] An example of this approach is the design and synthesis of affinity tagged enzyme substrate reagents 1 and 2 (Scheme 5) to simultaneously assay lysosomal β-galactosidase and N-acetyl-α-D-glucosaminidase, respectively. Deficiency of the former enzyme results in one of the lysosomal storage diseases, GM_1-gangliosidosis, a condition that occurs in the population with a frequency of about 1 in 50,000 and leads to early death of affected children. Deficiency of N-acetyl-R-D-glucosaminidase results in the rare lysosomal storage disorder Sanfilippo syndrome type B. This example has been described in Gerber et al., 1999, which is incorporated by reference herein in its entirety.

[0110] Conjugates 1 and 2 consist of biotin as an affinity tag, which is coupled to sarcosine. Biotin allows highly specific capture of the substrate conjugate through noncovalent binding to streptavidin immobilized on agarose beads (Bayer et al., 1990). Sarcosine provides an N-methylated amide linkage to biotin to block the enzyme biotinidase, which is often present in the cellular fluids and could cause cleavage of the conjugate molecule during the assay (Wilbur et al., 1997). In addition, it was found that biotinyl-sarcosine conjugates can be displaced from streptavidin by addition of biotin. The N-biotinylsarcosine block is linked to a polyether diamine, the length of which can be varied to avoid mass/charge overlaps of products and internal standards. The linker also allows facile introduction of multiple deuterium atoms (i.e., 8 deuteriums in 5 and 4 in 6, Scheme 5) to permit the synthesis of internal standards. The d8-linker was made by reacting $DOCH_2CH_2OCH_2CH_2OD$ with CD_2=CDCN in benzene with catalytic NaOD (Ashikaga, K., et al., 1988) and the resulting dinitrile was reduced to the diamine with Ra—Ni. The d4-linker was made in the same way using ethylene glycol and CD2=dCDCN in CH_3CN and catalytic NaOH.

[0111] In addition, the linker is hydrophilic to ensure good water solubility of the substrate conjugate, and it has basic groups which are efficiently protonated by ESI and thus ensure sensitive detection by mass spectrometry. The target carbohydrate substrates are attached to the polyether linker by a β-alanine unit (Scheme 5). The enzymatic product conjugates 3 and 4 are also shown Scheme 5. Conjugates 1 and 2 were prepared as shown in Scheme 5. All reagents were purified to homogeneity by reverse-phase HPLC and characterized by high-field 1 H NMR and ESI-MS. The substrate was linked to the diamine spacer by Michael addition of the latter onto the p-acryloylamidophenyl glycoside, (Romanowska et al., 1994) and the intermediate was coupled with the tetrafluorophenyl ester of N-biotinylsarcosine (Wilbur et al., 1997).

[0112] The ESI-MS assay of β-galactosidase and N-acetyl-R-D-glucosaminidase is based on enzymatic cleavage of the glycosidic bond to release monosaccharide and conjugates 3 and 4 (mass differences are 162 and 203 Da, respectively). In a typical procedure, 0.2 mM 1 and 0.3 mM 2 were ~~incubated~~ with sonicated cultured fibroblasts from individual patients with β-galactosidase deficiency and with fibroblasts cultured from unaffected people. After ~~incubation,~~ labeled internal standards 5 and 6 were added, and the biotinylated components were captured on streptavidin-agarose beads. Quantitative strepavidin capture efficiency from a cell homogenate was observed with model reagents. After purification by multiple washings to remove nonspecifically bound components, the biotinylated products were

incubated

incubation

released by free biotin, and the eluant was analyzed by ESI-MS. About 85% release of the biotinylated products was observed after ~~incubation~~ with excess biotin for 90 min. A blank was obtained by quenching the assay with all components present at time zero.

[0113] A typical procedure, cell protein (75 μg) in 15 μL of water was added to 15 μL buffer (0.1 M Na citrate, pH 4.25) containing 2 (0.3 mM) and 1 (0.2 mM, added 5 h after addition of cell protein). After ~~incubation~~ for 5.5 h at 37° C., the reaction was quenched by addition of 200 μL of 0.2 M glycine carbonate buffer, pH 10.3, and 5 and 6 (1 nmol each) were added. After centrifugation to remove cell debris, the supernatant was loaded onto a bed of streptavidin-agarose (7 nmol biotin binding capacity, Pierce) in a small filtration device (micro BioSpin, Bio-Rad). After 5 min, filtration was effected by centrifugation, and the gel bed was washed with 0.1% Triton X-100 (about 1 min ~~incubation~~, then spin) and then six times with purified water (Milli-Q, Millipore). Elution was carried out in 25 μL of 50% methanol containing 56 nmol of free biotin (1-10 h ~~incubation~~, then spin). ~~Filtrate was diluted 4-fold with 50%~~ methanol/water, and 1 μL was analyzed by ESI-MS.

[0114] The ESI-MS spectrum of the blank **(FIG. 6A)** is remarkably simple, showing peaks of the $(M+H)^+$ ions from reagents 1 and 2 (m/z 843 and 840), internal standards 5 and 6 (m/z 689 and 641), and trace amounts of products 3 and 4 (m/z 681 and 637). Ions due to clusters of biotin also appear in the spectrum but did not interfere with the analysis. The presence of nondeuterated products in the blank may be due to nonenzymatic substrate reagent hydrolysis during sample work up or to collision-induced dissociation of the substrate ion in the gas phase. A MS/MS spectrum of the (conjugate $1+H)^+$ ion at m/z 843 gave a prominent fragment of (conjugate $3+H)^+$ at m/z 681 (spectrum not shown). The ESI-MS spectrum of a sample ~~incubated~~ with cell homogenate from a healthy individual clearly shows the β-galactosidase product at m/z 681 and the N-acetyl-α-D-glucosaminidase product at m/z 637 **(FIG. 6B)**. Triplicate enzymatic reactions using cells from a healthy patient yielded a β-galactosidase specific activity of 51±3 nmol/h/(mg cell protein) and an N-acetyl-α-D-glucosaminidase specific activity of 1.4±0.3 nmol/h/mg. Time course studies confirmed that the initial reaction velocities were being measured. Values obtained with cells from six healthy individuals ranged from 36±4 to 68±3 nmol/h/mg for β-galactosidase and 0.9±0.05 to 2.3±0.4 nmol/h/mg for N-acetyl-α-D-glucosaminidase. In contrast, very little enzymatic product above the blank level (0.9±0.9 and 0.8±0.6 nmol/h/mg) was observed when cells from two patients with galactosidase deficiency were used, whereas N-acetyl-α-D-glucosaminidase activity is clearly detected **(FIG. 6C)**. These spectra were obtained with 0.75 μg of cell protein, corresponding to ~1000 fibroblasts. Thus the ESI-MS method has very high sensitivity for biomedical applications.

[0115] ESI-MS was carried out on a Finnigan LCQ ion trap instrument. Data were collected in full scan mode from m/z 625 to 875 by direct infusion at 1.5 μL/min. Specific activities were obtained from the ratio of product to internal standard ion peak areas (averaged over 30 scans).

[0116] The approach described for assaying enzymes using substrate reagents and ESI-MS can be broadly applied. The multiplex technique can be expanded to assay dozens or more enzymes simultaneously in a single reaction, obviating the need for multiple assays to assist in confirming diagnoses of rare disorders. The method can be used to measure several enzymes simultaneously when evaluating the rate of chemical flux through a specific biochemical pathway or for monitoring biochemical signaling pathways. The affinity tag-capture reagent method for isolation of affinity tagged reaction products and substrates from complex mixtures is technically simple and can be readily automated, particular when biotin-strepavidin is employed. Because of the high sensitivity of the ESI-MS detection employed, which requires only sub-microgram quantities of the substrate reagents per assay, the synthesis of several hundred substrate reagents on a low-gram scale becomes practical and economical. Since most enzyme active sites are exposed to solvent, it is possible to attach an affinity tagged linker to most enzyme substrates while preserving enzymatic activity. Scheme 6 provides the structures of several additional enzyme substrates, suitable for use in this method, indicating by arrows allowable positions for tag attachment sites. Allowable tag sites for additional enzyme substrates can be determined by review of X-ray crystal structures of enzyme-substrate or enzyme-substrate analog structures. Using a standard computer graphics program, available X-ray data and by attaching an extended chain butyl group (as a model for the affinity tagged linker) to potential tag attachment sites, suitable attachment sites that show there are no enzyme-atoms in van der Waals overlap with the model tag can be predicted.

[0117] Analogous methods to those described above can be applied to the analysis of enzymes associated with other Sanfillipo Syndromes (A, C and D). SFA is associated with heparin sulfamidase, SFC is associated with acetyl-CoA-alpha-glucosaminide N-acetyltransferase and SFD is associated with N-acetylglucosamine 6-sulfatase. Exemplary affinity tagged enzyme substrate reagents useful in the analysis of these enzymes and the diagnosis of these disorders are provided below. The methods can also be applied of the diagnosis of Niemann-Pick Type A and B disease by assaying for acid sphingomyelinase and to the diagnosis of Krabbe disease by assaying for galactocerebroside beta-galacatosidase. These enzymes are currently assayed employing fluorophore-derivatized reagents as indicated in Scheme 7. Enzyme substrate reagents for assay of these enzymes in the methods herein can be readily prepared by replacement of the fluorophore with an A-L group herein. This approach to preparation of affinity tagged enzyme substrates is generally applicable to any known fluorophore-derivatized enzyme substrate or substrate analog.

[0118] Table 4 provides exemplary enzymes that are associates with certain birth defects or disease states. These enzymes can be assayed by the methods described herein.

[0119] Assaying Enzymatic Pathways for Carbohydrate-Deficient Glycoprotein Syndromes (CDGS)

[0120] The methods and reagents of this invention can be employed to quantify the velocities of multiple enzymes pertinent to diagnosis of CDGS diseases.

[0121] CDGS Type Ia and Ib are caused by the deficiency or absence of the enzymes phosphomannoisomerase (PMIb) (Type Ib) and phosphomannomutase (PMM2) (Type Ia) which are part of a multistep pathway (Scheme 8) for

conversion of glucose to mannose-1-phosphate (Freeze, 1998). The monosaccharide substrates involved in the pathway are fructose-6-phosphate, mannose-6-phosphate, and mannose-1 -phosphate. These monosaccharides can be somewhat difficult to convert to substrate conjugates because it is not a priori clear which atom on the sugar should be conjugated with the linker without impairing enzyme activity. PMIb and PMM2 can, however, be assayed indirectly. Mammalian cell microsomes contain dolichol-P-mannose synthase which catalyzes the reaction of dolichol-phosphate with GDP-mannose to form dolichol-P-mannose and GDP (Scheme 8, Chapman et al. 1980). This synthase can be assayed using the methods of this invention, specifically with a biotin-linker substrate. Microbial PMM and the enzyme which makes GDP-mannose from GTP and mannose-1-P, GDP-mannose pyrophosphorylase, are readily purified from bacteria and yeast (Glaser, 1966, Preiss, 1966), and these enzymes can be supplied exogenously to the enzyme assay. If PMIb activity is deficient, little or no mannose-6-P will be made when the reaction sequence is started by addition of fructose-6-P. Without mannose-6-P, mannose-1-P and GDP-mannose will not be made, and thus no conjugated-dolichol-β-mannose will be detected by ESI-MS. Exogenous GTP is supplied as a requirement for the GDP-mannose pyrophosphorylase step, and ATP is omitted so that mannose-6-P cannot be made from mannose. To assay PMM2, the reaction sequence is initiated with mannose-6-P, and PMM2 deficiency results in the failure to make conjugated-dolichol-P-mannose.

[0122] The carrier dolichol is a ~60- to 105-carbon isoprenoid. Evidence is accumulating that many enzymes that operate on carbohydrates attached to dolichol chains are tolerant to significant shortening of the dolichol chain; even 10- and 15-carbon dolichols are tolerated (Rush and Wachter, 1995). It appears that such enzymes act on the water-soluble carbohydrate portion of the dolichol conjugate and thus have little or no requirement to bind the dolichol anchor. Based on this, an affinity labeled substrate for the direct assay of dolichol-P-mannose synthase and the indirect assay of PMIb and PMM2 is prepared by attaching an affinity labeled linker to the non-polar end of a short dolichol, such as the 10-carbon dolichol analog citronellol.

[0123] The synthesis of a biotinylated dolichol$_{10}$-substrate conjugate containing a sarcosinyl linker (B-S-Dol$_1$0-P) is shown in Scheme 9. Protected citronellol R=t-BuSiMe$_2$) is regioselectively oxidized at the terminal alyllic methyl group (McMurry and Kocovsky, 1984), and the allylic alcohol is coupled with biotinylsarcosine active ester R=CH$_3$). The citronellol 1-hydroxy group is subsequently deprotected and phosphorylated with $POCl_3$ (Rush and Wachter, 1995). In a parallel synthesis, d_5-sarcosine, CD_3NHCD_2COOH, is used to prepare the isotopically labeled (heavy) reagent for use as an internal standard. d_5-Sarcosine is readily prepares form commercially available materials ($BrCD_2COOD$ and CD_3NH_2) using standard synthetic techniques.

[0124] The deuterated internal standard, B-d_5-S-Dol$_{10}$-P-Mannose, is synthesized enzymatically by ineubating hen oviduct microsomes with GDP-mannose and the synthetic B-d_5-S-Dol$_{10}$-P substrate conjugate (Rush and Waechler, 1995). An added advantage of the B-S-conjugate is that it allows for a facile affinity purification of the microsomal mannosylated product by specific capture on agarose-streptavidin beads followed by elution with free biotin.

[0125] This method employing affinity tagged short dolichol analogues is generally applicable for assaying other enzymes that operated on dolichol anchored carbohydrates. Such an approach is useful for the subsequent identification of enzyme deficiencies present in other types of CDGS that have not been yet identified.

[0126] CDGS Type II results from defective GlcNAc transferase II (GlcNAc-T II) which transfers GlcNAc from UDP-GlcNAc to the 2-position of a mannose residue in the intermediate branched oligosaccharide (the Core Region) in the process of building up the disialo-biantennary chain (Scheme 10) (Schachter, 1986; Brockhausen et al, 1989). GlcNAc transferase II is one of the six known enzymes that mediate highly regiospecific glycosylation of the mannose residues in the Core Region. The Core Region is anchored at the reducing end to chitobiosylasparagine, where the asparagine residue is part of the peptide chain of the glycosylated protein. The latter structure unit in the substrate can be replaced by a hydrophobic chain without loss of enzyme activity (Kaur et al., 1991). Thus, the substrate conjugate for CDGS Type II is assembled by linking a affinity-labeled linker group to the reducing end to chitobiosylasparagine. However, the latter structure unit in the substrate can be replaced by a hydrophobic chain without loss of enzyme activity (Kaur et al., 1991). For example, commercially available α-D-manno-pyranosylphenyl-isothiocyanate can be coupled to a biotin-labeled linker and the 5,6-hydroxyls are selectively protected as illustrated in Scheme 11 (Paulsen and Meinjohanns, 1992). Coupling of the equatorial 3-OH with per-O-acetylmannosyl-1-trichloroacetamidate (Paulsen et al, 1993) will provide a disaccharide conjugate (Scheme 12). If a minor amount of coupling occurs at the axial 2-OH group the products can be separated by HPLC. After deprotection, the primary 6-OH is coupled with a second equivalent of per-O-acetylmannosyl-1-trichloroacetamidate to yield the Core Region conjugate. Deprotection of the O-acetyl groups yields the substrate conjugate for GlcNAc transferase I which can be converted to the GlcNAc-T II substrate by enzymatic glycosyl transfer using a Triton X-100 rabbit liver extract, a reaction that has been carried out on a preparative scale (Kaur et al., 1981).

"oh" not "zero"

[0127] The synthesis of the deuterium labeled derivative needed for the internal standard is performed in parallel by using a labeled PEG-diamine building block (Gerber et al., 1999). The biotinylated trisaccharide is converted to the tetrasaccharide (product of GlcNAc-T II) by ~~incubation~~ with ~~UDP~~-GlcNAc and transferase II ((Kaur and Hindsgaul, 1991; Tan et al., 1996) and utilizing the B-S handle for affinity ~~purification~~ of the enzymatic products.

incubation

UDP

[0128] CDGS Type V

[0129] The lipid-linked oligosaccharide (LLO) that is transferred to the Asn residue of the glycosylated protein is composed of 2 GlcNAc, 9 mannoses, and 3 glucoses. It has recently been shown that microsomes from CDGS type V patients are greatly deficient in the enzyme that transfers one or more glucose residues during LLO biosynthesis (Korner et al, 1998). Since the transferase that attaches the carbohydrate unit of LLO to the Asn residue discriminates against the glucose-deficient LLO, CDGS Type V patients have fewer numbers of carbohydrate units attached to glycopro-

teins, such as transferrin (Korner et al., 1998). However, the few carbohydrate units that are present are full-length, demonstrating that residual glucosyl transfer occurs in type V CDGS patients (Korner et al., 1998). Thus, quantification of the rate of Asn glycosylation by ESI-MS would constitute a viable assay of CDGS Type V syndrome.

[0130] Synthetic peptides with 3-7 amino acid residues containing the Asn-Xaa-Ser/Thr sequence have been shown to be good substrates for glycosylation (Ronin et al., 1981). The strategy for the ESI-MS assay of the oligosaccharide transferase relies on a B-S conjugate of an appropriate peptide containing the Asn-Xaa-Ser/Thr sequence (Scheme 13). A heptapeptide, NH_2-Tyr-Gln-Ser-Asn-Ser-Thr-Met-NH_2 (SEQ ID NO:1) has shown high activity in a previous study (Ronin et al., 1981). The peptide is readily available by standard peptide synthesis using an in-house automatic synthesizer. The heptapeptide and its glycoconjugates can be ionized by ESI to provide stable singly-charged ions. Coupling of BS-tetrafluorophenyl ester with NH_2-Tyr-Gln-Ser-Asn-Ser-Thr-Met-NH_2will directly yield the substrate for the transferase. Several products are expected from the enzymatic glycosylation and subsequent modifications of the oligosaccharide antenna. The products can be prepared enzymatically by ~~incubating~~ thyroid rough microsomes with BS-Tyr-Gln-Ser-Asn-Ser-Thr-Met-NH_2 and Dol-P-Glu (Ronin et al., 1981a), followed by affinity purification of the biotinylated products. Product distribution due to different degrees of glycosylation can be monitored by ESI-MS, and the major components can be purified by HPLC. An analogous procedure using a B-$N(CD_3)CD_2CO$-conjugate is used to prepare deuterated internal standards.

incubating

[0131] The molecular masses of the ionized substrate conjugates for the set of enzymes assayed for CDGS Ia, Ib, II, and V syndromes, as well as products and internal standards are compiled in Table 5, which shows that no isobaric overlaps among the $(M+H)^+$ species occur. The close spacing between the $(M+Na)^+$ ion from the Type Ia,b product and the $(M+H)^+$ ion of the demannosylated B-(N-C_2D_5)-2,2-D_2-Gly-Dol_{10}-P internal standard can be readily avoided by adjusting the ESI-MS conditions by addition of Na^+ ions to generate the gas phase ions as Na-adducts.

Na^+

[0132] All three of the targeted enzymes can be analyzed simultaneously in a single biological sample, such as a cell lysate. The PMM2 and PMIb cannot be assayed simultaneously because they require the addition of different exogenous substrates. Nevertheless, two assays using identical ESI-MS techniques can be used for diagnosing the various CDGS types instead of relying on a battery of different methods.

[0133] Clinical Enzymology Assays

[0134] A fibroblast cell pellet is thawed on ice. Sufficient 0.9% NaCl is added to give a final protein concentration in the lysate of ~5mg/mL (typically 100 mcL), and the cell pellet is sonicated in ice water 5 times for 2 seconds each at moderate power. Total protein is determined spectrophotometrically using the BCA reagent (BCA Protein Assay kit, Pierce).

[0135] The total enzyme reaction volume is 20- 30 mcL. The substrate stock solutions are maintained at concentrations of 3 mM (SFB) and 2 mM (GM1). These concentrations were measured by 1H-NMR signal integration versus an internal standard (formamide proton of DMF). Final concentration of substrates is 0.3 and 0.2 mM, respectively. A volume of reaction buffer (e.g. 200 mM sodium citrate, pH 4.5) equal to the difference of the substrate addition (2-3 mcL) plus sufficient cell sample volume to equal 50 û 75mcg total protein from 20-30 mcL is added to a 0.5 mL Eppendorf tube, followed by substrate. The sample is cooled on ice, and patient cell sample is added. The reaction is initiated by ineubation at 37° C.

[0136] For SFB: The reaction is allowed to proceed for 4.5-6 hours, after which GM1 substrate can be added or the reaction can be quenched with 100 mcL of 200 mM glycine-carbonate buffer, pH 10.5.

[0137] For GM1: The reaction is allowed to proceed for 0.5 hours. Quenching is as for SFB.

[0138] After quenching, the samples are placed on ice. Internal standards are added (1 nmol each, i.e. 50 mcL of a 0.02 mM solution). The samples are microfuged at ~15,000 rpm for 2 min at room temperature to pellet cell debris. Streptavidin-Agarose beads (Immunopure immobilized streptavidin, Pierce) are placed in a micro bio-spin chromatography column (Bio-Rad). Sufficient beads are added to give a total biotin binding capacity of 5 nmol (typical binding capacity 100 pmol per mcL of beads as determined by Pierce). The sample supernatant is transferred to the bio-spin tube and allowed to bind for 10 minutes at room temperature. The sample is spun at ~3,000 rpm to remove excess supernatant, then washed once with 0.01% Triton X-100 and at least five times with purified water, spinning the tube in-between to remove solution. For each wash, sufficient wash solution is added to fill the bio-spin tube.

[0139] The purified beads are then treated with 30 mcL purified water, followed by 10 mcL of a 4 mM biotin solution. The tubes are capped at the bottom to prevent leakage and allowed to ineubate at 2-8° C. for 2-12 hours. The samples are spun at ~3,000 rpm to elute the sample into a clean Eppendorf tube.

[0140] The sample is then diluted with 60 mcL of 50% methanol/water and infused into the ion-trap mass spectrometer. The ESI-MS spectrum is tuned to reduce non-specific cleavage of the samples by first analyzing a blank sample (cell lysate added after reaction quench). The infused sample is analyzed by ion chromatogram integration of a 1 amu-wide window about the $(M+H+)^+$ ions of product and internal standard.

[0141] Results are reported in nmol product formed/hour of ineubation/milligrams total protein in reaction mixtures.

[0142] Clinical Analysis of Patient Samples for GM1 and SFB

[0143] Patient skin fibroblasts were obtained as frozen pellets, and stored at û20° C. until use. Two GM1 affected samples and six normal controls were analyzed.

[0144] 50 mcL of 0.9% NaCl was added to each patient cell pellet. The pellets were lysed by sonication in ice water 5× for 2 seconds each at moderate sonication power, chilling the microtip in ice water in between sonications.

[0145] Samples were quantitated by BCA (Pierce) assay as follows:

[0146] Reagent A and B were mixed in 50:1 ratio as described. A protein standard curve was prepared using bovine serum albumin as a standard at concentrations of 2, 1, 0.5, 0.2, and 0.05 mg/mL. A portion of the patient sonicates were diluted 1:15 in water, and 5 mcL of each diluted patient sample and standard curve point was added to separate glass culture tubes containing 200 mcL water, in duplicate. The samples were then diluted with 1 mL of the mixed BCA reagent, vortexed to mix, and incubated at 37° C. for 60 minutes. The samples were allowed to cool to room temperature, and analyzed against a blank containing only 200 mcL water. The samples were analyzed by monitoring absorbance at 562 nm in polystyrene cuvettes. Average patient absorbance values were blank corrected and compared to standards via linear regression.

[0147] The patient protein concentrations were determined to be:

[0148] 1.(Affected) 12.2 mg/mL, 2. (Normal) 10.8 mg/mL, 3. (Normal) 11.9mg/mL, 4. (Normal) 12.1 mg/mL, 5. (Normal) 10.3 mg/mL, 6. (Normal) 7.79 mg/mL, 7. (Normal) 15.7 mg/mL, 8. (Affected) 11.4 mg/mL. Incubations were performed in a total of 30 mcL of total volume. The amount of reaction buffer (200 mM sodium citrate, pH 4.25) added to blank Eppendorf tubes was the difference of the substrate volume (3 mcL of each substrate stock solution, 2 mM for GM1 and 3 mM for SFB, for a total of 6 mcL) plus the volume of cell lysate required to equal 75 mcg total protein, from 30 mcL. For example, patient 1. incubation mixture initially contained 3 mcL of SFB substrate solution, 6.14 mcL patient cell lysate, and 17.86 mcL reaction buffer. The GM1 substrate was added later in the incubation (see below).

[0149] Each patient sample was analyzed in triplicate. The reaction mixtures were kept on ice during preparation, and the reaction was initiated by transfer to a 37° C. water bath. 5.5 hr into the incubation, 3 mcL GM1 substrate was added to each reaction, and after an additional 0.5 hours the reactions were placed on ice and quenched with 200 mcL of a 200 mM glycine-carbonate buffer, pH 10.25.

[0150] The purification and analysis procedures are as described in Clinical Enzymology Assay (Typical).

[0151] The resultant enzyme activities, as an average standard deviation nmol product/hour incubation/mg total protein:

	B-Gal		SFB	
	RATE	+/- SD	RATE	+/- SD
Normals				
Patient 2	68.0	2.6	0.90	0.05
Patient 3	35.5	3.9	1.54	0.38
Patient 4	51.1	2.7	1.36	0.26
Patient 5	38.8	8.3	1.01	0.12
Patient 6	51.4	9.9	2.25	0.36
Patient 7	40.9	3.7	1.12	0.20
Affecteds				
GM_1 (#1)	0.9	0.9	0.80	0.21
GM_1 (#8)	0.8	0.6	0.70	0.20

[0152] The following synthetic methods refer to Schemes 14-23. Synthesis for GM1-gangliosidosis (beta-D-galactosidase deficiency)

[0153] 1. 2,3,5,6-Tetrafluorophenyl trifluoroacetate (1) 25 g (0.15 mol) 2,3,5,6-tetrafluorophenol, 35 mL (0.2 mol) trifluoroacetic anhydride and 0.5 mL boron trifluoride etherate were refluxed for 18 hours under argon atmosphere. Trifluoroacetic anhydride and trifluoroacetic acid were removed by distillation at room temperature. The trifluoroacetic anhydride fraction was returned to the mixture, and the reaction was refluxed for 24 hours. This was repeated twice. After final distillation at room temperature, the desired product 1 was distilled at reduced pressure (62° C./45 mmHg) to produce a colorless liquid (30 g, 82%). 1 H-NMR. (Gamper, H. B., 1993). Biotin-2,3,5,6-tetrafluorophenyl ester (2) A 2.5 g (10.3 mmol) quantity of d-biotin in 20 mL anhydrous DMF under argon atmosphere was warmed to 60° C. with stirring to effect dissolution. 1.7 mL (12.5 mmol) triethylamine was added, followed by 3.4 g (12.5 mmol) 1. The mixture was stirred for 2 hours, after which the solvent was removed by rotary evaporation. The resultant semi-solid was triturated with 15 mL ether twice to produce a white solid (2.6 g, 65%). 1 H-NMR. (Wilbur, D. S., et al., 1997). N-methylglycylbiotinamide-methyl ester (3) A 2.5 g (6.4 mmol) quantity of biotin tetrafluorophenyl ester in 30 mL anhydrous DMF under argon atmosphere was added to a mixture of 1.1 g (7.7 mmol) N-methylglycine methyl ester hydrochloride dissolved in 10 mL anhydrous DMF and 1.25 mL (9.0 mmol) triethylamine. The reaction mixture was stirred at room temperature for 2 hours, then the solvent was removed by rotary evaporation. The residue was extracted with chloroform (2x100 mL), washed with water (2x20 mL), and dried with anhydrous sodium sulfate. The solvent was removed under vacuum to yield 2.1 g (98%) of methyl ester of N-methylglycine biotinamide as an oil. 1H-NMR. (Wilbur, D. S., et al., 1997).

[0154] 4. N-methylglycylbiotinamide acid (4) N-Methylglycylbiotinamide methyl ester was hydrolyzed in a mixture of 31 mL MeOH and 10 mL of 1N NaOH at room temperature with stirring for 1 hour. The mixture was diluted with 50 mL 50% MeOH/water and neutralized with cation exchange resin, hydrogen form (AG MP-50, BioRad). The solution was filtered, the resin washed (3x50 mL) with 50% MeOH/water, and the solvents removed by rotary evaporation to yield 1.6 g (90%) of N-methylglycylbiotinamide acid as an off-white solid. 1H-NMR. (Wilbur, D. S., et al., 1997).

[0155] 5. p-Acrylamidophenyl-β-D-galactopyranoside (5) 40 mg (0.15 mmol) p-aminophenyl β-D-galactopyranoside was added to 25 mL methanol and 200 mcL triethylamine with stirring. The solution was chilled in an ice bath. 53.3 mg (0.6 mmol) acryloyl chloride was dissolved in 5 mL dry methylene chloride and added dropwise to the stirred solution over 5 minutes. The reaction was allowed to return to room temperature, followed by 2 hours of stirring. The solution was then treated with successive anion and cation exchange resins (AG MP-1 and AG MP-50, respectively, Bio-Rad) until neutral pH was obtained with moist pH paper. Solvent was removed by rotary evaporation to

yield a solid (43 mg, 90%). 1 H-NMR. (Romanowska, A., et al., 1994). Michael addition product of 4,7,10-trioxa-1,13-tridecanediamine and 5 (6) 20 mg (0.07 mmol) 5 was added to a stirred solution of 80 mg (0.35 mmol) 4,7,10-trioxa-1,13-tridecanediamine in 5 mL 0.2M sodium carbonate, pH 10.5 at 37° C. The reaction was allowed to proceed for 3 days, after which the solution was neutralized with dilute trifluoroacetic acid and purified by reverse-phase HPLC (Vydac C-18 prep-scale column, 6 mL/min. Mobile phase: H_2O (0.08% TFA)/ACN (0.08% TFA)) to give 7.3 mg of product. (Romanowska, A., et al., 1994).

[0156] 7. GM1 substrate conjugate of 4 and 6 (7) A 2.5 mg (7.4 mcmol) quantity of 4 was dissolved in 1.5 mL anhydrous DMF with stirring, under argon atmosphere. 5 mcL triethylamine was added, followed by 2.3 mg (8.8 mcmol) 1. The formation of active ester was monitored by silica TLC (5:1 $CHCl_3/CH_3OH$, Rf 0.5, UV) by briefly drying the spotted TLC plate with a stream of air. After 25 minutes, the mixture was added to 3.2 mg (5.9 mcmol) 6 in 1 mL anhydrous DMF. After 2 hours, the solvent was removed by vacuum centrifugation and the final product was purified by reverse-phase HPLC (Vydac C-18 prep-scale column, 6 mL/min. Mobile phase: H2O (0.08% TFA)/ACN (0.08% TFA)). Yield 4.6 mg. (For analogous chemistry, see Wilbur, D. S., et al., 1997). 8. 1,2,10,11-octadeutero-3,6,9-trioxa-1,11-undecanedinitrile (8) 1 g (9.4 mmol) of diethylene glycol was dissolved in 2 mL D2O in a 10 mL round bottom flask under argon atmosphere. The D_2O was removed by rotary evaporation and the process was repeated 4 times. The d-2 diethylene glycol was additioned with 25 mL dry benzene, followed by 1.6 g (28.2 mmol) d-3 acrylonitrile with stirring under argon atmosphere. After 12 h, the solvent was removed under reduced pressure and the resultant semisolid was extracted with chloroform (2×5 mL). The solvent was removed by rotary evaporation to yield 1.85 g (89%) product. (Ashikaga, K., et al., 1988).

[0157] 9. 2,3,11,12-octadeutero-4,7,10-trioxa-1,13-tridecanediamine (9) Raney nickel (Aldrich) was washed five times with anhydrous methanol by inversion and decantation. 50 mg of the washed catalyst was placed in 20 mL anhydrous methanol, followed by 1 g (4.6 mmol) 8 in a 50 mL screw-cap vial fitted with a Teflon-lined rubber septum. The vial headspace was flushed for a few min with H_2 gas via an 18-gauge needle piercing the septum. The cap was screwed on tightly and the entire assembly was charged to 40 psi H_2 and placed in a hot water bath (80° C.) for 4 hours, after which the solid catalyst was removed by filtration and the methanol evaporated. The final product was purified by reverse-phase HPLC (Vydac C-18 prep-scale column, 6 mL/min. Mobile phase: H_2O (0.08% TFA)/ACN (0.08% TFA)). Yield 180 mg. (Ashikaga, K., et al., 1988).

[0158] 10. Deuterated analog of 6 (10) 25 mg (0.09 mmol) 5 was added to a stirred solution of 90 mg (0.4 mmol) 9 in 5 mL 0.2M sodium carbonate, pH 10.5 at 37° C. The reaction was allowed to proceed for 3 days, after which the solution was neutralized with dilute trifluoroacetic acid and purified by reverse-phase HPLC (Vydac C-1 8 prep-scale column, 6 mL/min. Mobile phase: H_2O (0.08% TFA)/ACN (0.08% TFA)). Yield 6 mg.

[0159] 11. Deuterated analog of 7 (11) A 3 mg (8.4mcmol) quantity of 4 was dissolved in 0.7 mL anhydrous DMF with stirring, under argon atmosphere. 5 mcL triethylamine was added, followed by 2.4 mg (8.9 mcmol) 1. The formation of active ester was monitored by silica TLC (5:1 $CHCl_3/CH_3OH$, Rf 0.5, UV) by briefly drying the spotted TLC plate with a stream of air. After 25 minutes, the mixture was added to 6 mg (11 mcmol) 10 in 1 mL anhydrous DMF. After2 hours, the solvent was removed by vacuum centrifugation and the final product was purified by reverse-phase HPLC (Vydac C-18 prep-scale column, 6 mL/min. Mobile phase: H_2O (0.08% TFA)/ACN (0.08% TFA)). Yield 1.8 mg.

[0160] 12. GM1 internal standard conjugate (12) 1.8 mg 11 was added to 2 mL of 100 mM Tris/10 mM MgCl2, pH 7.3 buffer with stirring. 15 units recombinant β-D-galactosidase (Sigma) was added, and after 12 hours the mixture was purified by reverse-phase HPLC (Vydac C-18 prep-scale column, 6 mL/min. Mobile phase: H_2O (0.08% TFA)/ACN (0.08% TFA)). Yield 1.5 mg.

[0161] Polyether Diamine Linker Synthesis (Second Generation)

[0162] Synthesis is based on chemistry previously described (Kataky, R. et. al., 1990), with minor modifications and an additional two steps. As an example, deviations from the established procedure as well as exact details for the additional steps are outlined below for the starting material diethylene glycol.

[0163] 1,11 [-Dicyano-3,6,9-trioxaundecane (13) To a stirred solution of 2% (w/v) sodium hydroxide (5 mL) and diethylene glycol (5.3 g, 50 mmol) was added acrylonitrile (7.95 g, 150 mmol). The mixture was stirred at room temperature overnight and additioned with 50 mL dichloromethane. The organic layer was washed 2× with brine and dried ($MgSO_4$). The solvent was removed by rotary evaporation. The oily residue was treated with 200 proof ethanol, and the solvent was removed by rotary evaporation. This was repeated 2× to remove excess unreacted acrylonitrile. The product was used without further purification

[0164] Diethyl 4,7,10-trioxatridecane-1,13-dioate (14) 2 g (9.4 mmol) 13, was dissolved in 5 mL ethanol. 1 g conc. sulfuric acid was added slowly, over 5 minutes. The reaction was heated to reflux overnight. The reaction was extracted with 40 mL methylene chloride, washed once with 10 mL water and 3× with 10 mL dilute brine solution. The organic layer was dried ($MgSO_4$) and solvent was removed to yield an oil. The final product was purified by silica chromatography (methylene chloride/ethyl acetate).

[0165] 1,13-dihydroxy-4,7,10-trioxatridecane (15) Prepared exactly as described, using tetrahydrofuran as solvent. (1.7 g, 5.5 mmol 14, 50 mL distilled [CaH_2] THF, 0.66 g, 16.5 mmol lithium aluminum hydride). Once addition was complete, excess LAH was quenched with ethanol, and the salts precipitated by dropwise addition of saturated sodium sulfate solution until a white precipitate formed. The solvent was removed, the precipitate washed 6×30 mL with THF

0.1 g dry potassium hydroxide in 20 mL acetonitrile, followed by 1.4 g (24 mmol) d-3 acrylonitrile with stirring overnight at room temperature. The reaction was filtered and the solvent was removed by rotary evaporation to yield an oil. Final product was purified by silica chromatography (chloroform/methanol) to yield a colorless oil 0.9 g (65%).

[0180] 20. 1,9-tetradeutero-3,6-dioxa-1,9-nonanediamine (26) Raney nickel (Aldrich) was washed five times with anhydrous methanol by inversion and decantation. 20 mg of the washed catalyst was placed in 30 mL anhydrous methanol, followed by 0.5 g (3 mmol) 25 in a 50 mL screw-cap vial fitted with a Teflon-lined rubber septum. The vial headspace was evacuated with H_2 gas via an 18-gauge needle piercing the septum. The cap was screwed on tightly and the entire assembly was charged to 40 psi H_2 and placed in a hot water bath (80° C.) for 4 hours, after which the solid catalyst was removed by filtration and the methanol evaporated. The final product was purified by reverse-phase HPLC (Vydac C-18 prep-scale column, 6 mL/min. Mobile phase: H_2O (0.08% TFA)/ACN (0.08% TFA)). 21. Deuterated analog of 23 (27) 20 mg (0.07 mmol) p-acrylamidophenyl β-D-galactoside was added to a stirred solution of 90 mg (0.4 mmol) 26 in 5 mL 0.2 M sodium carbonate, pH 10.5 at 37° C. The reaction was allowed to proceed for 3 days, after which the solution was neutralized with dilute trifluoroacetic acid and purified by reverse-phase HPLC (Vydac C-18 prep-scale column, 6 mL/min. Mobile phase: H_2O (0.08% TFA)/ACN (0.08% TFA)). Yield 2 mg.

[0181] 22. Deuterated analog of 24 (28) A 2 mg (6.3mcmol) quantity of 4 was dissolved in 1.5 mL anhydrous DMF with stirring, under argon atmosphere. 5 mcL triethylamine was added, followed by 2.1 mg (7.6 mcmol) 1. The formation of active ester was monitored by silica TLC (5:1 $CHCl_3/CH_3OH$, Rf 0.5, UV) by briefly drying the spotted TLC plate with a stream of air. After 35 minutes, the mixture was added to 4 mg (7mcmol) 27 in 1 mL anhydrous DMF. After 2 hours, the solvent was removed by vacuum centrifugation and the final product was purified by reverse-phase HPLC (Vydac C-18 prep-scale column, 6 mL/min. Mobile phase: H_2O (0.08% TFA)/ACN (0.08% TFA)). Yield 1.2 mg.

[0182] n23. SFB internal standard conjugate (29) 1.2 mg 28 was added to 2 mL of 100 mM Tris/10 mM MgCl2, pH 7.3 bufferwith stirring. 15 units recombinant β-D-galactosidase (Sigma) was added, and after 12 hours the mixture was purified by reverse-phase HPLC (Vydac C-18 prep-scale column, 6 mL/min. Mobile phase: H_2O (0.08% TFA)/ACN (0.08% TFA)). Yield 0.7 mg.

[0183] Clinical Substrate Synthesis for Sanfilippo Syndrome, type D (a sulfatase deficiency).

[0184] 24. p-Acrylamidophenyl-α-D-N-acetylglucosamine-6-sulfate (30) 100 mg (0.28 mmol) 20 was added to 10 mL dry DMF under argon atmosphere with stirring at room temperature. 89 mg (0.56 mmol) sulfur trioxide-pyridine complex was dissolved in 2 mL dry DMF and was added to the reaction in 0.7×, 1.1×, 1.3× and 1.9× equivalents (+700 mcL, +400 mcL, +200 mcL, and +600 mcL). The reaction progress was monitored by 1H-NMR shift of the anomeric (C1) proton chemical shift from 5.29 to 5.24 ppm by removal of 15 mcL of solution 1 hour after addition of each amount of sulfating reagent. The removed mixture was dried by vacuum centrifugation and redissolved in d-6 DMSO and analyzed. Upon the appearance of more than two forms (starting material and C-6 sulfate) of the C1 anomeric proton, the reaction was removed to −20° C. and stored. The product was purified by vacuum centrifugation to remove solvent, followed by reverse-phase HPLC (Vydac C-18 prep-scale column, 6 mL/min. Mobile phase: H_2O (0.08% TFA)/ACN (0.08% TFA)). Yield 72%.

[0185] 25. Michael addition product of 18 and 30 (31) 25 mg (0.058 mmol)30 was added to a stirred solution of 83 mg (0.35 mmol) 18 in 5 mL 0.2M sodium carbonate, pH 10.5 at 37° C. The reaction was allowed to proceed for 3 days, after which the solution was neutralized with dilute trifluoroacetic acid and purified by reverse-phase HPLC (Vydac C-18 prep-scale column, 6 mL/min. Mobile phase: H_2O (0.08% TFA)/ACN (0.08% TFA)). Yield 10 mg.

[0186] 26. SFD substrate conjugate of 4 and 31 (32) A 5.7 mg (0.018 mmol) quantity of 4 was dissolved in 1.0 mL anhydrous DMF with stirring, under argon atmosphere. 20 mcL dry triethylamine was added, followed by 5.5 mg (0.020 mmol) 1. The formation of active ester was monitored by silica TLC (5:1 $CHCl_3/CH_3OH$, Rf 0.5, UV) by briefly drying the spotted TLC plate with a stream of air. After 25 minutes, the mixture was added to 10 mg (0.015 mmol) 31 in 1 mL anhydrous DMF. After 2 hours, the solvent was removed by vacuum centrifugation and the final product was purified by reverse-phase HPLC (Vydac C-18 prep-scale column, 6 mL/min. Mobile phase: H_2O (0.08% TFA)/ACN (0.08% TFA)). Yield 5.4 mg.

[0187] 27. 1,2,14,15-octadeutero-1,15-diamino-5,8,11-trioxapentadecane (33) as referenced in Polyether Diamine Linker Synthesis, Second Generation.

[0188] 28. Deuterated analog of 31 (34) 25 mg (0.07 mmol) 20 was added to a stirred solution of 100 mg (0.4 mmol) 11 in 5 mL 0.2M sodium carbonate, pH 10.5 at 37° C. The reaction was allowed to proceed for 3 days, after which the solution was neutralized with dilute trifluoroacetic acid and purified by reverse-phase HPLC (Vydac C-18 prep-scale column, 6 mL/min. Mobile phase: H_2O (0.08% TFA)/ACN (0.08% TFA)). Yield 7 mg.

[0189] 29. SFD internal standard conjugate (35) A 4 mg (12.6 mcmol) quantity of 4 was dissolved in 1 mL anhydrous DMF with stirring, under argon atmosphere. 20 mcL triethylamine was added, followed by 4 mg (14 mcmol) 1. The formation of active ester was monitored by silica TLC (5:1 $CHCl_3/CH_3OH$, Rf 0.5, UV) by briefly drying the spotted TLC plate with a stream of air. After 20 minutes, the mixture was added to 7 mg (11 mcmol) 34 in 1 mL anhydrous DMF. After 4 hours, the solvent was removed by vacuum centrifugation and the final product was purified by reverse-phase HPLC (Vydac C-18 prep-scale column, 6 mL/min. Mobile phase: H_2O (0.08% TFA)/ACN (0.08% TFA)). Yield 2.7 mg.

[0190] N-(d-Biotinyl-sarcosinyl)-12-aminododecanoic acid (36). Compound 4 (32.2 mg, 0.102 mmole) was dried overnight in vacuo (with P_2O_5). Dry DMF (2 mL) was added and the mixture was stirred with warming to affect dissolution under nitrogen.

[0191] Triethylamine (34 mcL) was added followed by 1 (20.4 mcL, 0.115 mmole) added in two 10.2 mcL portions, 5 min apart. The mixture was stirred for 1 hr at room temperature under nitrogen. 12-Aminododecanic acid (24.1 mg, 0.112 mmole, Sigma) was added in one portion, and the mixture was stirred at room temperature for 2 hr under nitrogen. $CHCl_3$ (80 mL) was added, and the organic solution was washed with two 10 mL portions of 1 m HCl. $CHCl_3$ was removed by rotary evaporation, and residual DMF was removed by vacuum centrifugation. The compound was dissolved in methanol and purified by HPLC (Vydac 218TP prep column). Solvent program is: 0-10 min, water with 0.06% TFA; 10-55 min, 0-100% methanol with 0.06% TFA, flow rate is 6 mL/min. Yield 31.7 mg. 1 H-NMR. ESI-MS, calculated 513.4, observed 513.4 $(M+H)^+$

[0192] N-hydroxysuccinimidyl ester of 36 (37). Compound 36 (9.8 mg, 19 mcmole) is dissolved in 100 mcL of dry DMF under nitrogen. N-hydroxysuccinimide (2.2 mg, 19 mcmole) was added followed by dicyclohexylcarbodiimide (3.9 mg, 19 mcmole). The mixture was stirred at room temperature for 60 h in the dark. Solvent was removed by vacuum centrifugation, and the residue was submitted to flash chromatography on silica gel using a gradient of $CHCl_3/CH_3OH$ (15/1) to $CHCl_3/CH_3OH$ (12/1). Yield 9.8 mg. 1H-NMR. ESI-MS, calculated 610.8, observed 609.7 $(M+H)^+$.

[0193] N-(N-(d-Biotinyl-sarcosinyl)-12-aminododecanoyl)-pyschosine (38). Compound 37 (6.2 mg, 10 mcmole) and pyschosine (4.7 mg, 10 mcmole, Sigma) were dissolved in 200 mcL of dry DMF under nitrogen. Diisoproylethylamine (5 mcL) was added, and the mixture was stirred under nitrogen for 2 days in the dark. The compound was injected directly onto the HPLC column (Vydac 218TP semi-prep), and the column was developed at 2 mL/min with 0-20 min, water with 0.06% TFA, then 20-80 min, 0-100% methanol with 0.06% TFA. Yield 3.8 mg. 1H-NMR. ESI-MS, calculated 957.3, observed 956.8 $(M+H)^+$.

[0194] N-(N-(d-Biotinyl-sarcosinyl)-1,2-aminododecanoyl)-sphingosylphosphorylcholine (39). Sphingosylphosphorylcholine (4.0 mg, Sigma) was mixed with 1 mL dry DMF and solvent was removed by vacuum centrifugation. This was repeated two more times. The final dried residue weighed 2.5 mg (5.4 mcmole). To this residue was added 3.3 mg of 37 (5.4 mcmole), 150 mcL of dry DMF, and 2.5 mcL of diisoproylethylamine. The mixture was stirred under nitrogen in the dark for 3 days. The compound was injected directly onto the HPLC column (Vydac 218TP semi-prep), and the column was developed at 2 mL/min with 0-20 min, water with 0.06% TFA, then 20-80 min, 0-100% methanol with 0.06% TFA. Yield 3.8 mg. 1H-NMR. ESI-MS, calculated 960.3, observed 958.7 $(M+H)^+$.

[0195] Conjugate of d-biotin with 1,13-diamino-4,7,10-trioxatridecane (40). Compound 2 was reacted with 1,13-diamino-4,7,10-trioxatridecane (Fluka) essentially as described for the synthesis of 3. The product was purified by HPLC (Vydac 218TP, semi-prep) using 0-100% methanol with 0.06% TFA over 30 min at 1.5 mL/min.

[0196] Iodoacetylated 40 (41). Compound 40 was treated with 5 equivalents of iodoacetic anhydride (Aldrich) in dry DMF with stirring under nitrogen for 4 h at room temperature. The product was purified on HPLC as for 40. The structure was confirmed by ESI-MS.

[0197] Octadeuterated 41 (42). The title compound was prepared as for the 40 using 9 instead of 1,13-diamino-4,7,10-trioxatridecane.

[0198] Octadeuterated 42 (43). The title compound was prepared from 42 as for 41. The structure was confirmed by ESI-MS.

[0199] Exemplary MS^N Techniques and Instrumentation

[0200] An automated LC-MS/MS system for the identification of proteins by their amino acid sequence has been developed. A schematic representation is shown in **FIG. 7** The system, which consists of an autosample, a capillary HPLC system connected on-line to an ESI triple quadrupole MS/MS instrument and a data system is operated in the following way: Proteins (typically separated by 1D or 2D gel electrophoresis) are cleaved with a specific protease, usually trypsin. the resulting cleavage fragments are placed in an autosampler. Every 37 minutes the autosampler injects one sample into the HPLC system and the peptides are separated by capillary reverse-phase chromatography. As separated peptides elute from the chromatography column, they are ionized by the ESI process, enter the MS and the mass to charge ratio (m/z) is measured. Any peptide ion whose intensity exceeds a predetermined intensity threshold is automatically selected by the instrument and collided in the collision cell with inert gas. These collisions result in peptide fragmentation, primarily at the bonds of the peptide backbone (collision induced dissociation, CID). The masses of the CID fragments are measured and recorded in the data system. The CID spectrum of a peptide contains sufficient information to identify the protein by searching sequence databases with the uninterpreted MS/MS spectra. This is accomplished with the Sequent program. The program identifies each peptide in a sequence database which has the same mass as the peptide that was selected in the MS for CID and predicts the MS/MS spectrum for each one of the isobaric peptides. By matching the experimentally determined CID spectrum with computer generated theoretical CID spectra, the protein from which the observed peptide originated is identified. The system is capable of analyzing protein samples in a fully automated fashion at a pace of less than 40 min. per sample. Since each peptide represents an independent protein identification and usually multiple peptides are derived from one protein, protein identification by this method is redundant and tolerant to proteins co-migrating in a gel. The system is well suited for the detection and characterization of modified residues within polypeptide chains. The LC-MS/MS technique and automated analysis of the generated CID spectra can be used for the methods of this invention.

[0201] Identification of Proteins at Sub-femtomole Sensitivity by Solid-phase Extraction Capillary Electrophoresis Tandem Mass Spectrometry (SPE-CE-MSIMS)

[0202] Protein identification by this method is based on the same principle as described above, except that peptide separation and ionization are performed at significantly higher sensitivity. **Fig. 8** shows a schematic representation of the key design elements. The design of the system and its mode of operation have been published. Peptides derived from protein digests are concentrated by SPE, separated by CE and analyzed by ESI-MS/MS. The resulting uninterpreted CID spectra are used to search sequence databases

with the Sequest software system. The SPE extraction device is a small reversed-phase chromatography column of the dimensions 0.18×1 mm which is directly packed in a fused silica separation capillary. Peptides contained in a sample solution are adsorbed and concentrated on the SPE device, eluted in an estimated 100-300 nl of organic solvent and further concentrated by electrophoretic stacking and/or isotachophoresis to an estimated volume of 5-30 nl. The peptides are then separated by CE in a 20 μm or 50 μm i.d. capillary and directly ionized by ESI as they leave the capillary. With this system, peptide masses can be determined at a sensitivity of 660 attomoles (approx. 500 fg for a 20 residue peptide) at a concentration limit of 33 amol/μl and that proteins can be identified by the CID spectra of automatically selected peptides at less than 10 fmol (0.5 ng for a protein of 50 kDa) of sample at a concentration limit of less than 300 amol/μl. this technique is used for the analysis at very high sensitivity of the peptide samples generated by the experiments. It has also been demonstrated that the analysis time available for automated CID experiments can be significantly extended by data-dependent modulation of the CE voltage. If several peptide ions are detected coincidentally in the MS, the CE voltage is automatically dropped. This results in a reduction of the electroosmotic flow out of the capillary and therefore in an extension of the time period available for selecting peptide ions for CID. The net effect of this peak parking technique is an extension of the dynamic range of the technique because the increased time available is used for CID of ions with a low ion current Once all the peptide ions are analyzed, electrophoresis is automatically reaccelerated by increasing the CE voltage to the original value.

TABLE 1

Relative, redundant quantitation of α-lactalbumin abundance (after mixing with known amount of the same protein with cysteines modified with isotopically heavy biotinylating reagent)

Peptide #	m/z (light)	Charge state	Peptide Sequence	Ratio (heavy: light)
1	518.4	2+	(K) IWCK	2.70
2	568.4	2+	(K) ALCSEK (SEQ ID NO:2)	2.68
3	570.4	2+	(K) CEVFR (SEQ ID NO:3)	2.90
4	760.5	2+	(K) LDQWLCEK (SEQ ID NO:4)	2.82
5	710.1	3+	(K) FLDDDLTDDIMCVK (SEQ ID NO:5)	2.88
6	954.2	3+	(K) DDQNPHSSNICNISCDK (SEQ ID NO:6)	2.90
7	1286.9	4+	(K) GYGGVSLPEWVCTTFHTSGYDT QAIVQNNDSTEYGLFQINNK (SEQ ID NO:7) (SEQ ID NO.	NA*

*Isotope ratio was not analyzed because on a 4⁻ peptide the isotope patterns were highly overlapping due to differences of only 2 amu between heavy and light ions.

[0203]

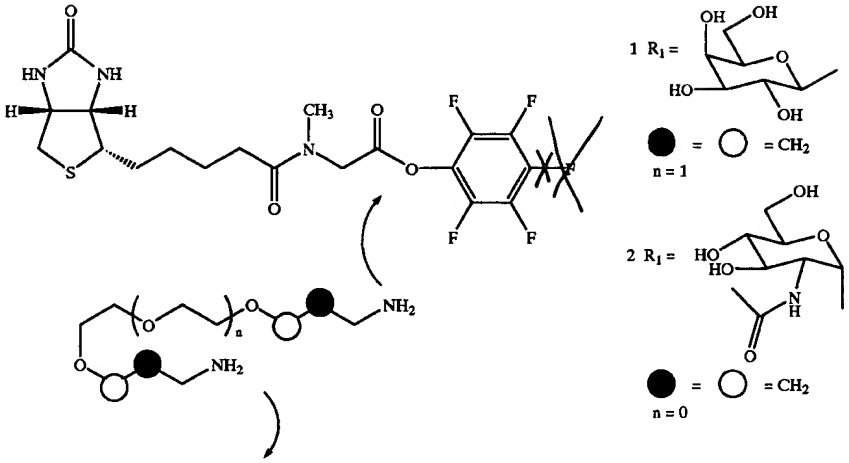
TABLE 2

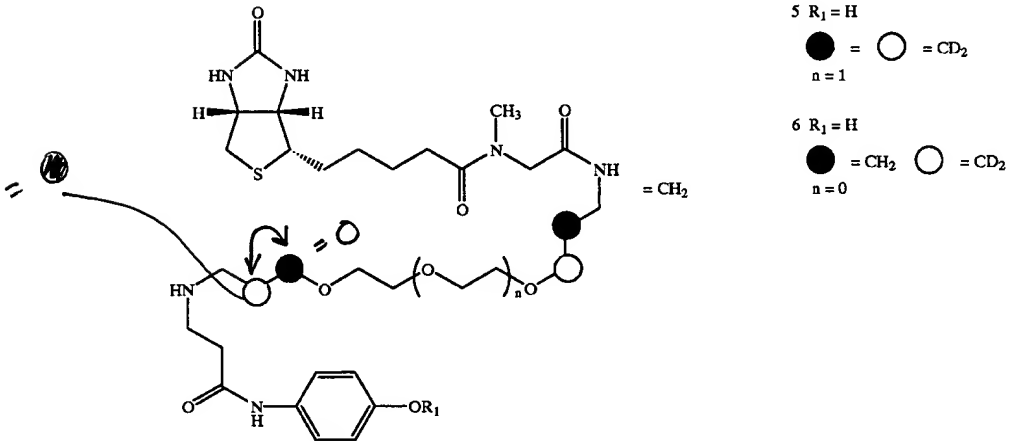
Sequence identification and quantitation of the components of a protein mixture in a single analysis.

Gene Name*	Peptide sequence identified	Observed ratio (d0/d8)+	Mean ± SD	Expected ratio (d0/d8)⁻	% error
LCA_BOVIN	ALC#SEK (SEQ ID NO: 8	0.94	0.96 ± 0.06	1.00	4.2
	C#EVFR	1.03			
	FLDDLTDDIMC#VK (SEQ ID NO: 9)	0.92			
OVAL_CHICK	ADHPFLFC#IK (SEQ ID NO: 10)	1.88	1.92 ± 0.06	2.00	4.0
	YPILPEYLQC#VK (SEQ ID NO: 11)	1.96			
BGAL_ECOLI	LTAAC#FDR (SEQ ID NO: 12)	1.00	0.98 ± 0.07	1.00	2.0
	IGLNC#QLAQVAER (SEQ ID NO: 13)	0.91			
	IIFDGVNSAFHLWC#NGR (SEQ ID NO: 14)	1.04			
LACB_BOVIN	WENGEC#AQK (SEQ ID NO: 15)	3.64	3.55 ± 0.13	4.00	11.3
	LSFNPTQLEEQC#HI (SEQ ID NO: 16)	3.45			
G3P_RABIT	VPTPNVSVVDLTC#R (SEQ ID NO: 17)	0.54	0.56 ± 0.02	0.50	12.0
	IVSNASC#TTNC#LAPLAK (SEQ ID NO: 18)	0.57			
PHS2_RABIT	IC#GGWQMEEADDWLR (SEQ ID NO: 19)	0.32	0.32 ± 0.03	0.33	3.1
	TC#AYTNHTVLPEALER (SEQ ID NO: 20)	0.35			
	WLVLC#NPGLAEIIAER (SEQ ID NO: 21)	0.30			

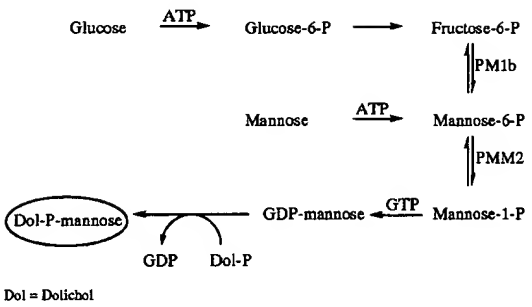
*Gene names are according to Swiss Prot nomenclature (www.expasy.ch).
+Ratios were calculated for each peptide as shown in FIG. 3.
⁻Expected ratios were calculated from the known amounts of proteins present in each mixture.
ICAT-labeled cysteinyl residue.

Scheme 5
Structures of Substrate Conjugates 1 and 2, Product Conjugates 3 and 4, and Internal Standards 5 and 6

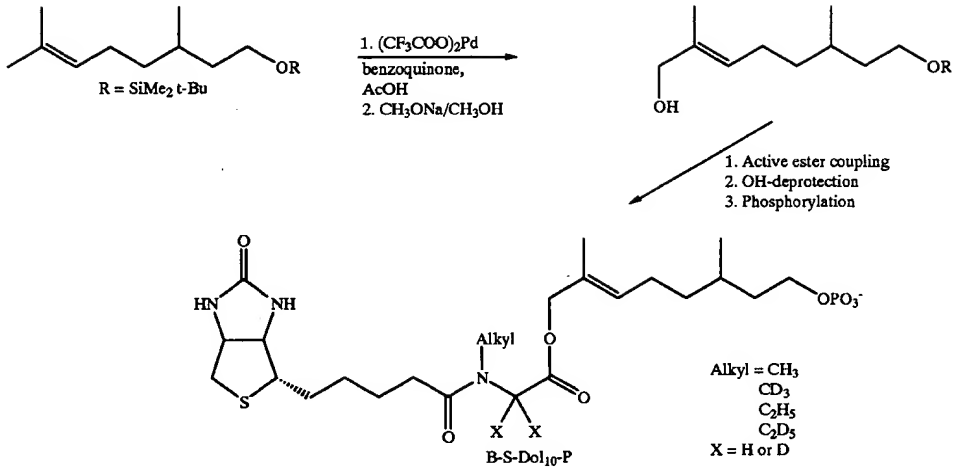




Scheme 8



Scheme 9



Scheme 10